Repressing Effects of Sodium Dodecyl Sulfate on Solvent-Gelatinization of Urine Samples

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The present work aimed to use a small volume of solvent for extraction of a large volume of urine sample in order to reduce the increase in hazardous waste. Sodium dodecyl sulfate (SDS) was found to exert an ability to repress gelatinization of solvent, which could be caused by shaking a small volume of solvent with a large volume of urine sample. Use of SDS provided the following simple method. Filtrate (100 ml) of a human urine sample (pH 9—10 or 3—4) was spiked with 100 μl of 5% SDS solution, and shaken with 2.5 ml of chloroform. The clear, separated chloroform layer was dried in a glass tube (2 ml), and the residue dissolved in solvent (chloroform: 50 μl) and subjected to analysis. This characteristic behavior of SDS for separation of clear chloroform was interpreted by hypotheses deduced from chemical equilibria among SDS, protein, and chloroform.

Key words —— sodium dodecyl sulfate, urine sample, chloroform, liquid–liquid partition extraction, deproteinization

INTRODUCTION

Solid–liquid phase extraction[1] using a cartridge is convenient for extraction of a small volume of urine sample (less than 10 ml), however, it fails for large volumes because of its small capacity. The conventional liquid–liquid partition method[2–4] is suitable for a large volume of urine sample, however, a large amount of solvent is required to extract a large volume of sample, because small amounts of solvent are often gelatinized by a large volume of urine. The excessive use of solvent results in much waste. This is contrary to the current social trend to reduce hazardous waste. The present work purposed to use a small volume of solvent[5] for extraction of a large volume of urine sample. The problem of solvent gelatinization was in part solved by the previous method[6]: urinary gelatinizing material[7] was removed by filtration of urine samples which had been treated with metaphosphoric acid at pH 3.5 or with ferric chloride at pH 9—10. However, this treatment reduced the recovery of phenolic chemicals. The present study found that the gelatinization phenomenon was avoidable by adding a certain amount of sodium dodecyl sulfate (SDS) to the urine sample. The optimum amount of SDS was examined for extraction of a large volume of urine sample with a small volume of chloroform, and applied to extraction of some chemicals[8] spiked in a human urine sample.

Considering why SDS exerts such a characteristic ability to repress gelatinization of chloroform, the present work attempted to suggest a hypothesis from binding theory of SDS and protein.[9–12] At the same time, the forming ability and surface tension were measured to evaluate guess how much surface activity remained in urine samples when SDS displayed such a repressing property.

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MATERIALS AND METHODS

Materials —— Human urine sample: 4—5 l for each experiment, was collected from several persons at 4℃ within one week and immediately used, unless otherwise mentioned. Old urine sample: this was prepared by leaving standing a collected urine sample for more than one month at 4℃, allowing generation of mucous materials. Urinary gelatinizing material (UGM) was

prepared by the previous method[7]. Ovalbumin, sodium dodecyl sulfate (SDS), and Triton X-100 were purchased from Wako Pure Chemical Industries Ltd. Osvan (10% content of benzalkonium chloride) from Takeda Pharmaceutical Industries Ltd. Diposh-A from Environmental Science Center Ltd. Mild Fresh (ingredient: polyoxyethylene alkylether, fatty acid alkanolamide, linear alkylbenzene sulfonate (LAS), *etc.*) from Mitsuei Ltd. Mama Lemon (LAS, alkylether sulfonate, *etc.*) from Lion Ltd. Attack Kao (LAS, polyoxyethylene alkylether, alkylether sulfonate, fatty acid sodium salt, *etc.*) from Kao Ltd. Standard solution of estazolam: 1 μg/μl in methanol, which was prepared by extracting one tablet of Eurodin (1 mg estazolam content, Takeda Pharmaceutical Industries Ltd.).

Measurement of Foaming Ability——Sample solution (10 ml) was added to a test tube (15 mm i.d., 18 cm long), and spiked with different amounts of SDS solution as indicated on the abscissa of the figure. The mixture was shaken, and the height (cm) of the foam generated was read 10 min after shaking.

Measurement of Surface Tension (ST)——Magnitude of ST was represented by the height of sample solution risen in a capillary tube (0.8 mm i.d., 12 cm long, Nippon Rigaku Kikai Ltd.) according to the formula $\Gamma = r\rho gh/2$ (CGS unit): Γ, surface tension (dyne/cm); r, radius; ρ, density; g, gravity acceleration; h, height of solution in the tube.

Measurement of Volume of Chloroform Recovered——Clear chloroform layer (and gel layer) obtained after shaking with sample solution was pipetted into a flat-bottomed glass volumeter (4.0 mm i.d., 20 cm long). The length of each layer was read with a scale, and converted to volume with a coefficient of 13.3 μl/mm in height.

Extraction Method——Urine sample (pH 9—10 or 3—4) was filtered through a paper (a Toyo Roshi No. 101, 24 cm i.d., 16-pleat type). The filtrate (100 ml) was spiked with 100 μl of 5% SDS solution, and shaken with 2.5 ml of chloroform in a glass vessel (*e.g.*, a capable 200-ml container for instant coffee, 5 cm W × 5 cm D × 10 cm H). The clear chloroform layer was passed through a paper (a Toyo Roshi No. 526, 12 mm i.d.) which was inserted in a glass pipe (10 mm i.d., 10 cm long) and mounted with anhydrous sodium sulfate (*ca.* 100 mg). The filtrate was dried in a 2-ml glass tube under a current of air. The residue was dissolved in chloroform (50 μl) washing the wall of the tube with it. The whole solution was subjected to thin-layer chromatography.

Thin-Layer Chromatography (TLC)——The whole extract of a human urine sample (100 ml) with chloroform (2.5 ml) was applied, as one spot, on an ordinary phase silica gel plate (20 cm × 20 cm, Merck). This was developed with a solvent system of chloroform–methanol–28% ammonia solution (95 : 5 : 0.5, v/v/v) and stained with Dragendorff reagent and 20% sulfuric acid. The spot was densitometrically measured on a Shimadzu chromatoscanner CS-9000.

RESULTS AND DISCUSSION

Relation between Foam Height of SDS-Spiked Urine and Separability of Chloroform at pH 9.0

As shown in the upper panel of Fig. 1, each foam height curve X, Y, or Z gently increased up to 100 μl of 5% SDS solution, and rapidly rose at 300 μl. Under these conditions, addition of more than 50 μl of 5% SDS began to clarify the chloroform layer. Addition of 100 μl fully clarified the chloroform layer, as shown in the right area of curve A. Vigorous shaking of the gel-like chloroform layer shifted curve A (at 50 μl) to curve B (at 20 μl). Curve B was shifted to curve C by dehydrating the gel-like chloroform layer with anhydrous sodium sulfate: gel layer (g) in the left area of curve B was clarified. A maximal separation of chloroform was achievable by addition of 100 μl of 5% SDS solution to 100 ml of urine sample (pH 9.0). This addition point fell in the range of a gentle increase in foam height (upper panel). A flat broken line failed to specify any SDS range effective in extraction because of no extraction of urinary indicatory material.

Relation between Foam Height of SDS-Spiked Urine and Separability of Chloroform at pH 3.5

The same experiment was carried out using a pH-3.5 normal urine sample as in the above. Every foam height curve X, Y, or Z gently increased up to 70 μl of 5% SDS, and rapidly rose in the upper panel of Fig. 2. Clear chloroform layer appeared under curve A. Additional vigorous shaking of gel-like chloroform layer enlarged the clear chloroform area to under curve B. A full separation of clear chloroform was achieved by adding 100 μl of 5% SDS solution to 100 ml of urine sample (pH 3.5), as well as in the pH 9.0. This addition point fell in the range where the foam-height began to rise (upper panel), and gave a maximum photoabsorbance of extract (*i.e.*, maximum extraction efficiency) as shown by the

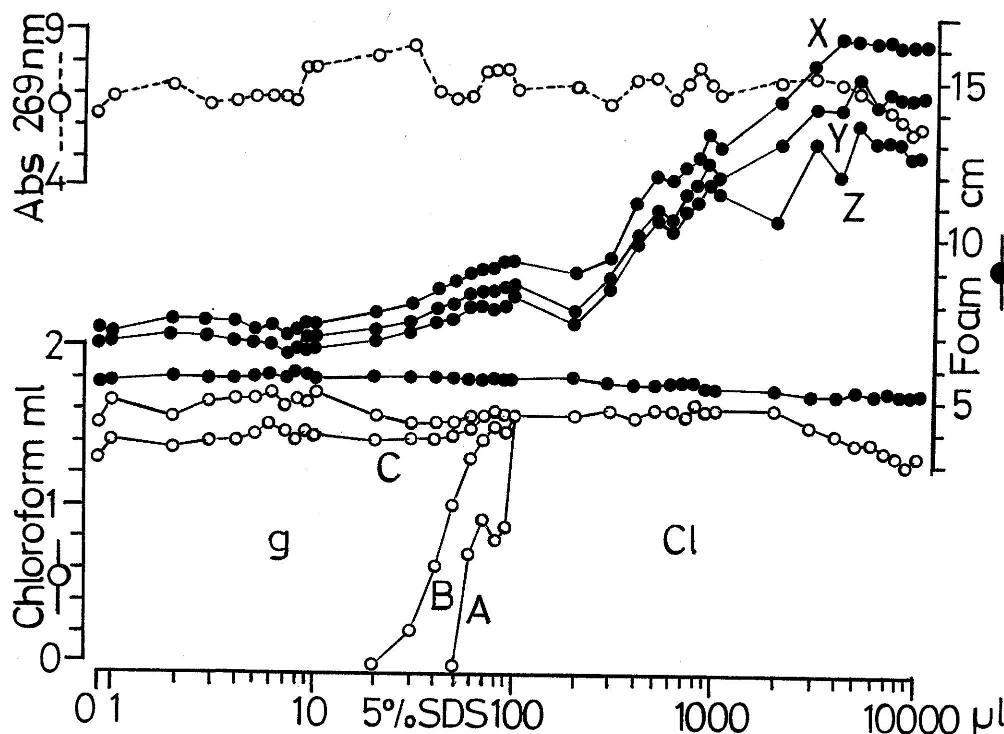

Fig. 1. Relation between Foam Height of SDS-Spiked Urine Sample and Separability of Chloroform at pH 9.0

Sample preparation: filtrate (100 ml, pH 9.0) of human urine + different amounts of 5% SDS. Curves X, Y, and Z: foam height 10, 60, and 180 min, respectively, after shaking (10 s) of the SDS–urine (10 ml) in a test tube (15 mm i.d., 18 cm long). Linear curve at a level of 5—6 cm: height of urine layer in the tube. Curve A: clear chloroform layer (Cl) and gel layer (g) 10 min after shaking (10 s) of the SDS–urine (100 ml) with chloroform (2.5 ml) in a vessel (5 cm W × 5 cm D × 10 cm H). Curve B: Cl successively obtained after shaking (10 s) of g (beside curve A) and Cl (under curve A) without urine layer. Curve C: Cl obtained by shaking g (beside curve B) with anhydrous sodium sulfate (*ca.* 200 mg). Broken line: photoabsorbance (at 269 nm) of Cl under curve C in 3 ml methanol.

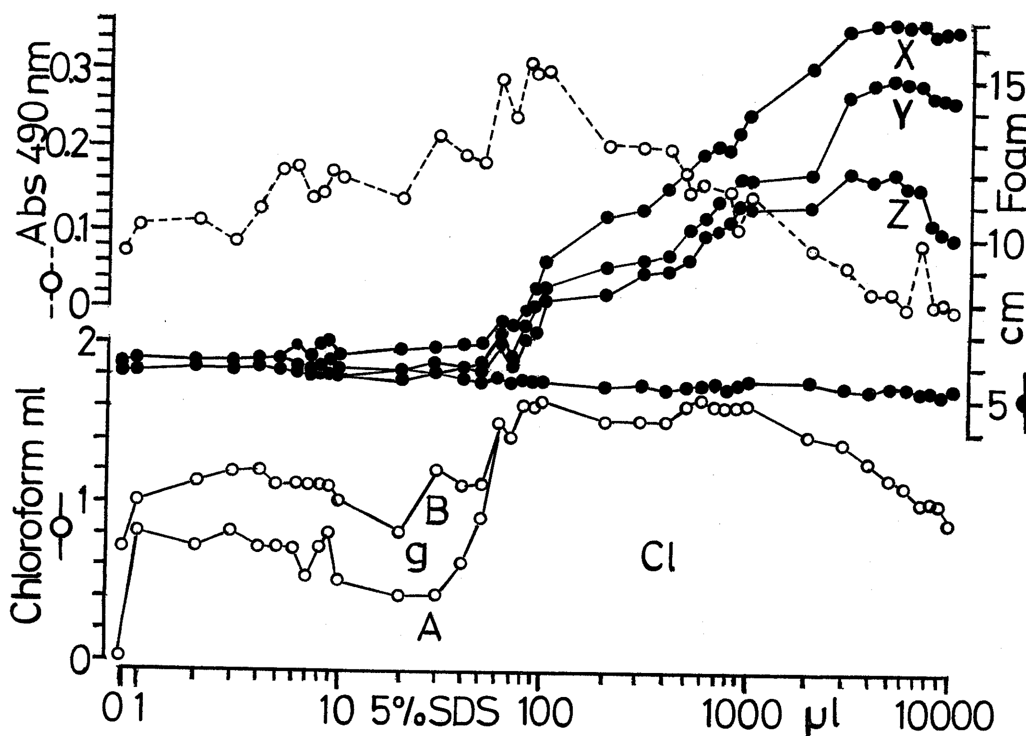

Fig. 2. Relation between Foam Height of SDS-Spiked Urine Sample and Separability of Chloroform at pH 3.5

Method: as in the legend to Fig. 1. Sample preparation: urine (100 ml, pH 3.5) + SDS (different amounts). Curves X, Y, Z, A and B: the same as in the legend to Fig. 1. Broken line: photoabsorbance (at 490 nm) of clear chloroform layer under curve B in 3 ml methanol.

broken line.

Effects of SDS on Separation of Chloroform for Extraction of Estazolam Spiked in Urine Sample

As shown in the upper panel (solid line) of Fig. 3, the foam-length (in a 200-ml vessel) gently increased up to 1000 μl of 5% SDS solution, and rapidly rose. The area under curve A demonstrated that clear chloroform was highly recoverable at addition of 400 through 1000 μl of 5% SDS to 100 ml of urine sample. This high recovery was achieved in a region of gentle increase in foam height. The clear chloroform area was enlarged to under curve B by shaking the gel-like chloroform layer. The broken line (upper panel) shows that estazolam was efficiently extractable in the chloroform, which was initially separated by addition of 90 through 2000 μl of 5% SDS. However, the material was not extracted in the later separated chloroform (under curve B), which had been gelatinized in the region of less than 90 μl and more than 3000 μl of 5% SDS. The present experiment used an old urine sample enriched with mucous materials generated through storage. Therefore, the difference in pattern between Fig. 3 and Fig. 1 (or Fig. 2) was attributed to the urinary quality. The irregular density peak, in the broken line, seemed to be caused by digression of spot from the correct lane on the TLC plate. However, the TLC-densitometry was convenient for monitoring the range of SDS effective for this extraction.

Comparison of Urinary Gelatinizing Material Solution with Ovalbumin Solution

Comparison (Fig. 4) was done at pH 3.5. As shown in panel (1), a pure water sample spiked with different amounts of SDS gradually foamed, up to addition of 50 μl of 5% SDS solution, and then vigorously foamed. Chloroform shaken with the sample was gelatinized with an increase in foam height over the range of more than 50 μl. As shown in panel (2), 0.01% UGM solution also gradually foamed up to 20 μl of 5% SDS, and then vigorously foamed. Chloroform was gradually clarified up to 10 μl of 5% SDS, and began to be gelatinized at the beginning of vigorous foam-

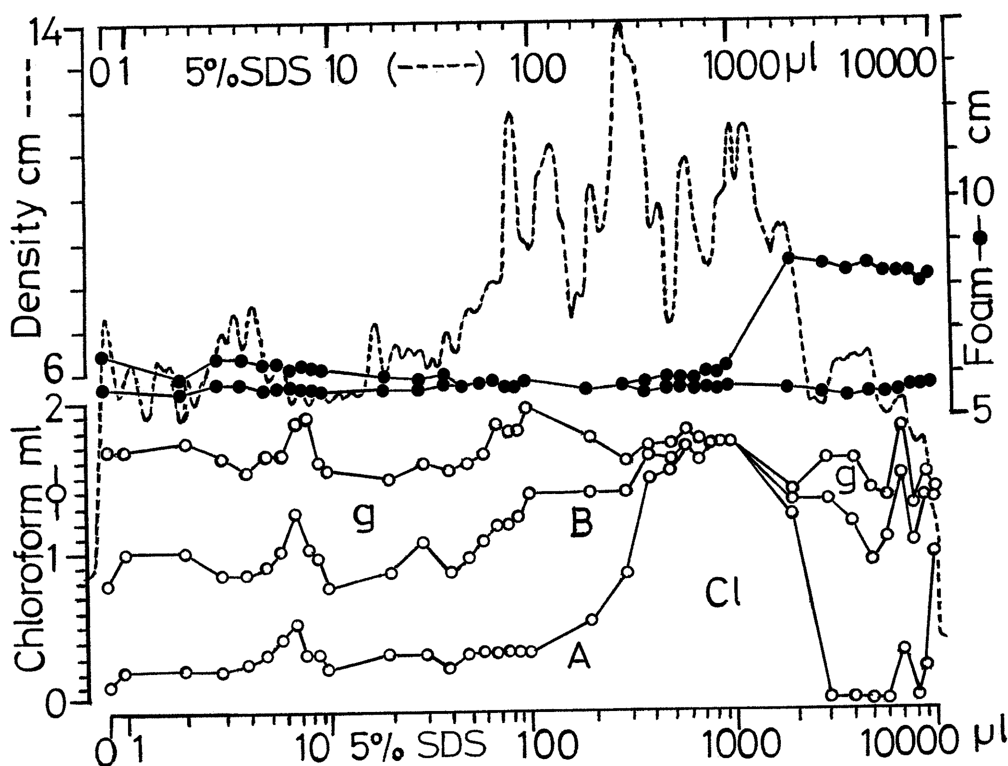

Fig. 3. Effects of SDS on Separation of Chloroform for Extraction of Estazolam Spiked in Urine Sample

Method: as in the legend to Fig. 1. Sample preparation: filtrate (100 ml, pH 9.0) of old urine sample (stored for over one month at 4℃) + 5 μg of estazolam + SDS (different amounts). Solid line in upper panel: foam height 10 min after shaking (10 s) the sample. Curves A and B: the same as in the legend to Fig. 1. Broken line in upper panel: densitogram obtained by scanning along a line of TLC-estazolam-spots offered by clear chloroform layers of curve B.

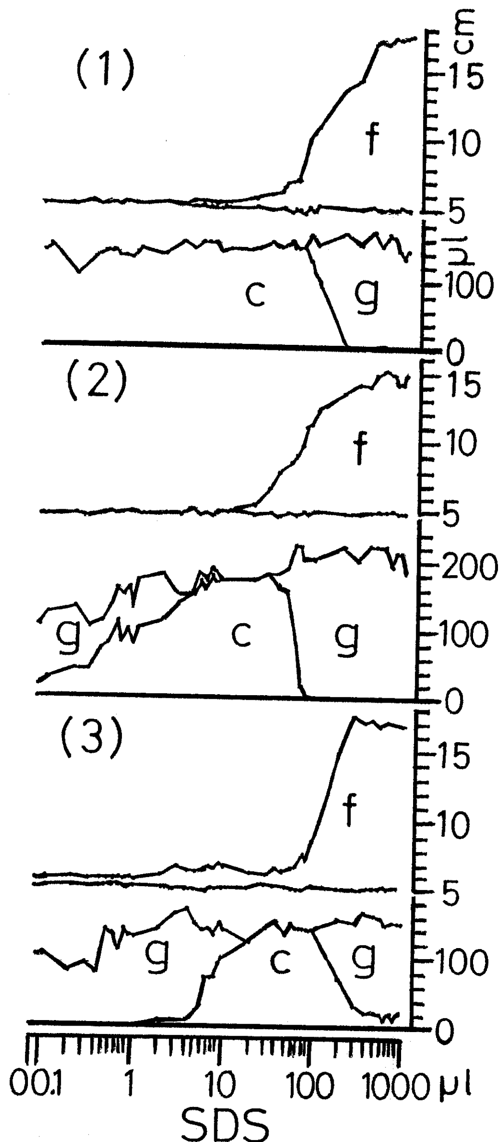

Fig. 4. Comparison of Urinary Gelatinizing Material Solution with Ovalbumin Solution at pH 3.5

Method: as in the legend to Fig. 2. Experimental scale: 1/10 th the scale of Fig. 2 using a test tube (15 mm i.d., 18 cm long) and 250 μl of chloroform. Sample solution: (1) pure water, (2) 0.01% urinary gelatinizing material (UGM), and (3) 0.01% ovalbumin. Sample preparation: each sample solution (10 ml, pH 3.5) + SDS (different amounts). Upper solid line in each sample: foam height (f) 10 min after the shaking. Lower curves in each sample: volumes of clear chloroform layer (C) and gel layer (g) 10 min after the shaking.

ing (30 μl of 5% SDS). As shown in panel (3), 0.01% ovalbumin solution also gradually foamed up to 100 μl of 5% SDS, and vigorously foamed. Chloroform was gradually clarified over the range of 1 to 5 μl, and rapidly clarified up to 20 μl. The chloroform began to be gelatinized at the beginning of vigorous foaming (100 μl). The first gelatinization of chloroform (g), which was observed at a low addtion of SDS for panels (2) and (3), seemed to be caused by an excess amount of protein compared with that of SDS added. The second gelatinization (g) observed at high addition of SDS seemed to be caused by the excess amount of SDS compared with that of protein. A delta zone of clear chloroform layer (c) was provided not only by UGM solution, but also by ovalbumin solution. The pure water sample failed to offer the zone because of no protein. Appearance of the delta zone (c) suggested that an equivalent mixing of protein and SDS should eliminate each other's ability to gelatinize chloroform. Ovalbumin and UGM gave a similar separation profile. This meant that UGM was physicochemically similar to ovalbumin. For the generation of delta zone, a protein-like material was needed. The same experiment was done at pH 9.0, as shown in Fig. 8. Panel 1-2 (UGM) and panel 2-2 (ovalbumin) show delta zone and trapezoidal zone, respectively. The SDS range, which gave clear chloroform, corresponded to the range of gentle increase in foam length (panel 1-1 right and 2-1 right) or corresponded to the range of gentle decrease in surface tension (panels 1-1 left and 2-1 left).

Effects of Surfactant Goods on Separation of Chloroform

As shown in Fig. 5, (1) Osvan (cationic surfactant) and (2) Triton X-100 (non ionic surfactant) faild to show a significant delta zone of clear chloroform layer (C). (3) Diposh-A showed a faint clear chloroform zone at high amounts. (4) Mama Lemon, (5) Mild Fresh, and (6) Attack Kao showed the delta zone. These three goods contain an anionic surfactant as the main ingredient. Therefore, anionic surfactant seemed necessary for the appearance of delta zone. SDS was found to be much more superior than any of the other surfactants tested here.

Effects of pH on Recovery of Chloroform with SDS

As shown in panel A of Fig. 6, pH adjustment to 4.5, 5.0, 5.5, 11.5, and 12 gelatinized a portion of the chloroform added. The latter two gel layers (pH 11.5 and 12) were clarified by shaking with clear chloroform layer after removal of the urine layer, as shown in panel B. However, the former three failed to be clarified (recovery of chloroform used: 46, 50, and 50%, respectively). Adjustment to pH 1 did not gelatinize chloroform, however it resulted in low recovery (48%). This

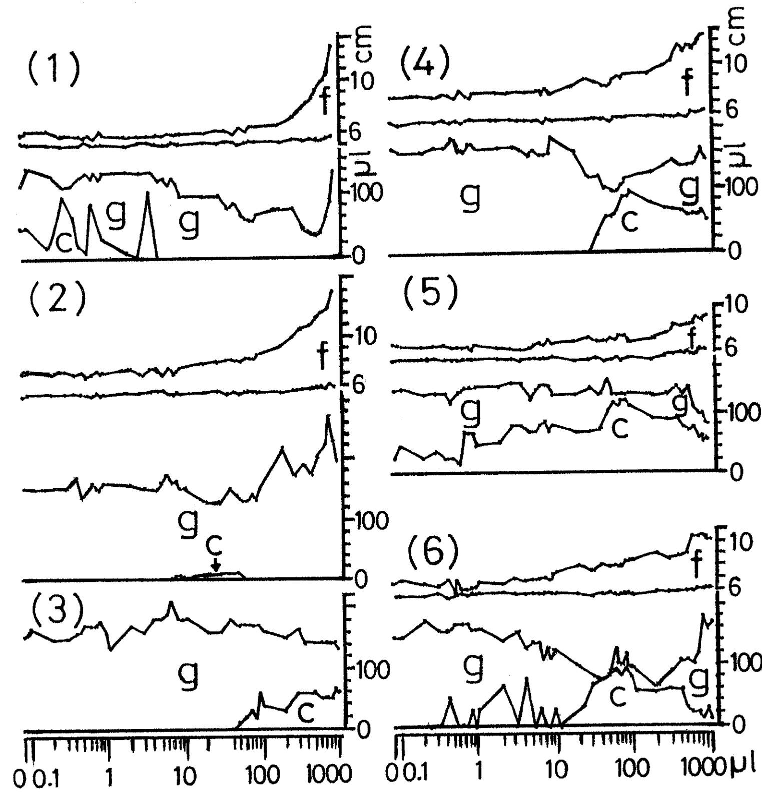

Fig. 5. Effects of Surfactants on Separation of Chloroform

Method: as in the legend to Fig. 4. Surfactants: (1) Osvan, (2) Triton X-100, (3) Diposh-A, (4) Mama Lemon, (5) Mild Fresh, (6) Attack Kao. Sample preparation: urine sample (10 ml, pH 3.5) + 5% surfactant solution (different amounts). Upper solid line and lower curves in each sample: the same as in the legend to Fig. 4.

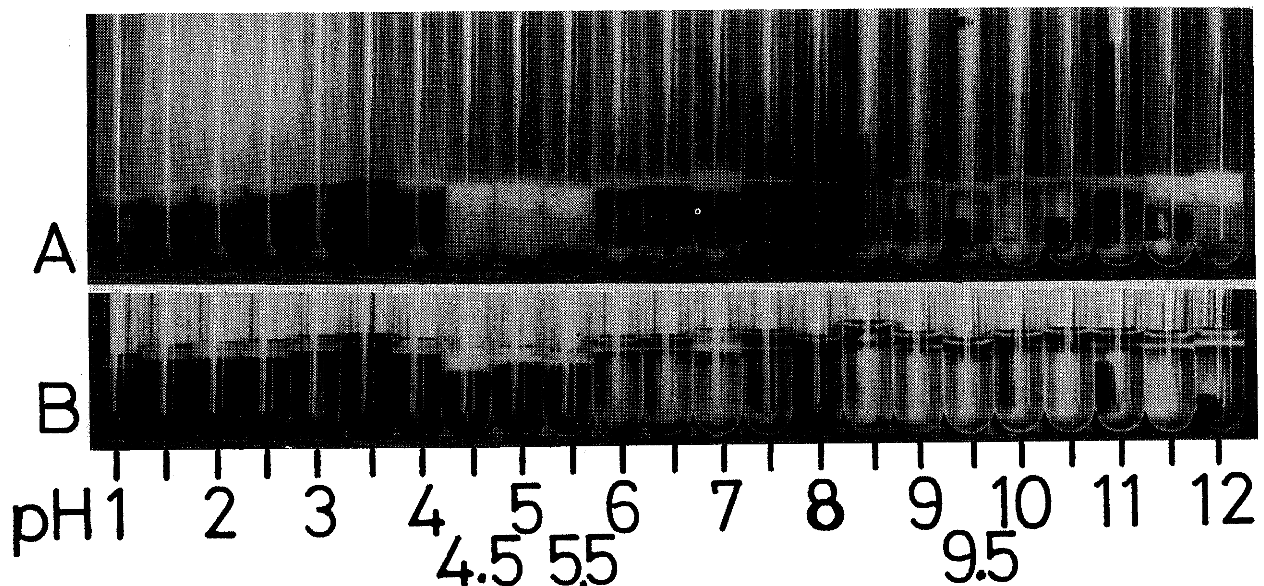

Fig. 6. Separation Profiles of Chloroform after Shaking with SDS-Spiked Urine Sample at Different pH Values

Method: as in the legend to Fig. 1. Sample preparation: different pH urine sample (100 ml; filtrate for pH 7.5—12 urine) + 100 μl of 5% SDS. Photography A: gel and clear chloroform layers, and remaining urine layer, which were transferred to a test tube (10 mm i.d., 10 cm long) 30 min after shaking. Photography B: gel and clear chloroform layers successively obtained by shaking the layers after removal of the remaining urine layer.

should be attributable to the increase in solubility of chloroform in urine. The other pH adjustments resulted in 55—65% recovery. Consequently, the pH adjustment to 3.5 was employed for extraction of acidic materials.

Recovery of Estazolam Added to Urine Sample

The present method was applied to the extraction of estazolam as an example. Table 1 shows the recoveries of estazolam obtained by single extraction. More than 60% recovery was achieved for a concentration of 5 or 10 μg estazolam in a 100-ml urine sample. A concentration as low as 2.5 μg estazolam in 100-ml urine sample offered more than 50% recovery.

Effects of SDS on Recovery of Chloroform

As shown in Fig. 7, the recovery of chloroform rapidly rose from 0.7 ml up to 2.0 ml, and then gently increased. Therefore, the present method employed 2.5 ml or 250 μl of chloroform for extraction of 100 ml or 10 ml of urine sample, respectively.

Hypotheses for Support of the Experimental Curve

For the recovery curve of chloroform (Fig. 7), the following hypothesis was constructed: chloroform S binds with urinary gelatinizing material B to form a complex SB in a mole ratio $m : n$, respectively. The hypothesis was for-

Table 1. Recovery of Estazolam Added to Urine Samples

Estazolam (μg) in urine 100 ml	Amount (μg) detected	Recovery (%) mean±S.D.
2.5	1.36	60.4
	1.57	69.8
	1.26	56.0
	1.36	60.0
	1.58	70.2
		63.3±5.7%
		n=5
5.0	2.85	63.3
	3.56	79.1
	3.31	73.6
	2.82	62.7
	2.72	60.4
	2.79	62.0
	2.76	61.3
	3.73	82.9
		68.2±8.4%
		n=8
10	6.89	76.6
	6.87	76.3
	8.33	92.6
	6.59	73.2
	4.55	50.6
		73.9±13.4%
		n=5

Method: as in the legend to Fig. 1. Sample preparation: filtrate (100 ml, pH 9.0) of urine + estazolam (2.5, 5.0, or 10 μg) + 100 μl of 5% SDS. Quantification: the whole extract obtained with 2.5 ml chloroform was subjected to TLC-densitometry.

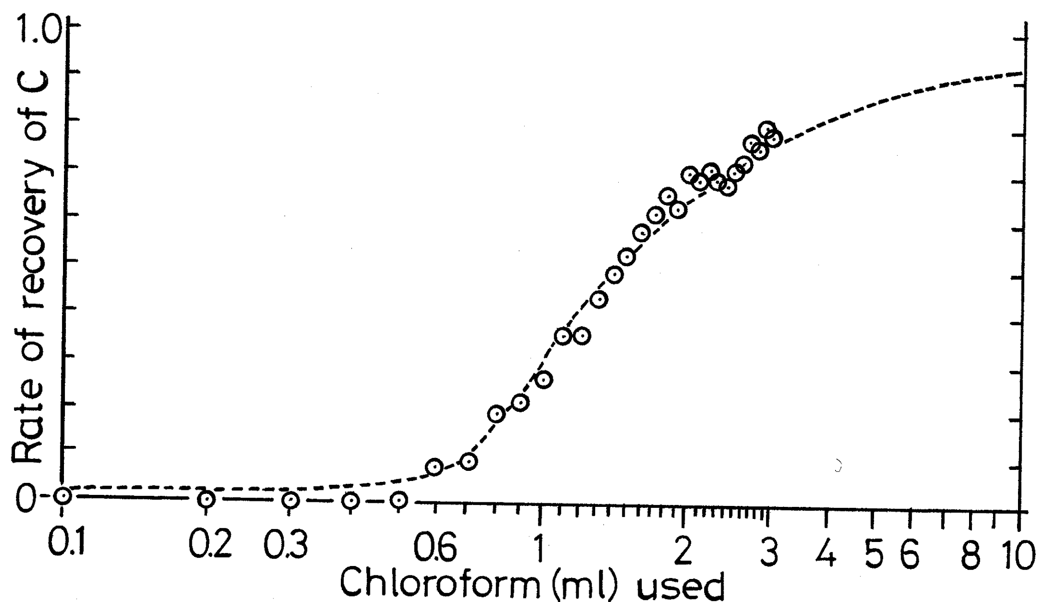

Fig. 7. Effects of SDS on Recovery of Chloroform

Method: as in the legend to Fig. 1, except the use of a uniform amount of 5% SDS (100 μl) and different amounts of chloroform. Open circle: rate (Y) of recovered chloroform (C) against that used (X). Broken line: curve depicted by theory, as detailed in the text.

mulated as follows: $[SB]/[S][B]=K$ (equilibrium constant), $[S]+[SB]\ m/(m+n)=X$, $[B]+[SB]\ n/(m+n)=$constant, and $[S]/X=Y$. These formulae were summarized as follows:

$$Y=(1/X)\ \mathrm{SQR}\ \{X/a+(b-X/2)^2\}-b/X+0.5 \quad (1)$$

Y=recovery rate of chloroform. X=volume (ml) of chloroform used. Constants $a=77.13$ and $b=0.7316$ were obtained by substituting two points of data: $X=0.6$, $Y=0.07$; and $X=1.0$, $Y=0.3$ into formula (1). Substitution of different values of X into formula (1) gave a broken line (Fig. 7) passing through the data spots. This coincidence of the experimental curve with the calculated one implied that the recovery of chloroform was interpretable by the present hypothesized equilibrium reaction.

For the surface tension curve (panel 1-1 left in Fig. 8), the hypothesis was as follows: SDS (S) binds with water W to make a complex WS, and $[WS]/[W][S]=K$ (equilibrium constant), $[S]+[WS]=V$(amount of SDS added: μl), $[S]=$ amount of free SDS, $[W]+[WS]=A$ (amount of water used), $[W]$=amount of free water, $[WS]=$ amount of WS. These formulae were rearranged as follows:

$$(A-[W])/[W](V-A+[W])=K \quad (1),$$

$$[W]=\mathrm{SQR}[\{(V-A+1/K)/2\}^2+A/K]-(V-A+1/K)/2 \quad (2),$$

H (surface tension ratio)$=\{M$ (height of solution in capillary tube at a volume $V)-B$(height of solution in the tube at $V=1000)\}/\ \{T$ (height of solution in the tube at $V=0)-\mathrm{B}\}$ (3). [W] was further hypothesized to be comparable to value

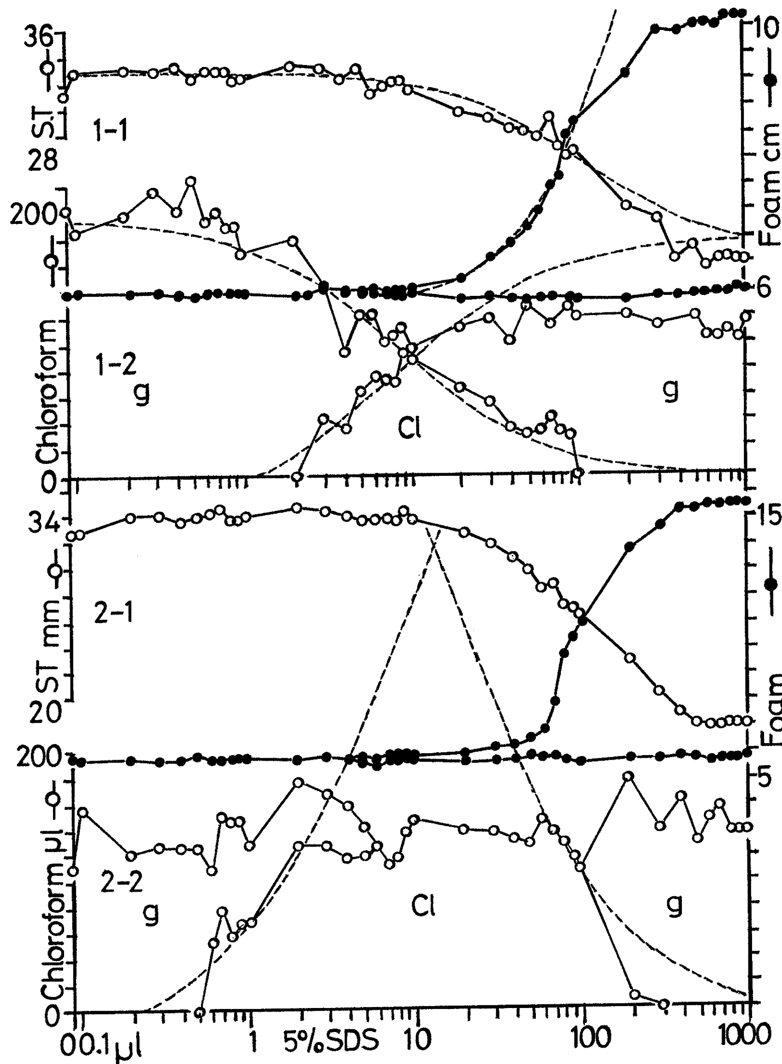

Fig. 8. Comparison of UGM Solution with Ovalbumin Solution at pH 9.0 and Curve Fitting by Hypothesis

This figure (pH 9.0) is a duplicate of Fig. 4 (pH 3.5). Method: as in the legend to Fig. 4, except for difference in pH of sample solution. Curve in 1-1-right and curves in 1-2: corresponding to (2)-UGM in Fig. 4. Curve in 2-1-right and curves in 2-2: corresponding to (3)-ovalbumin in Fig. 4. Broken lines: curves depicted by theory, as detailed in the text. ST (surface tension): left curves in 1-1 and 2-1.

H: [W]$=H$. This hypothesis changed the formula (2) to $M=[\mathrm{SQR}\ \{(V-A+1/K)^2/4+A/K\}-(V-A+1/K)/2](T-B)+B$ (4). Constant value $K=8.79102\times10^{-3}$ was obtained by substituting data ($T=33$ mm, $B=18$ mm, and $M=26$ mm at $V=100\ \mu$l) into the formula (3), and by substituting the calculated value H (=[W]), $V=100\ \mu$l, and $A=1$ into formula (1). Different values of M were obtained by substituting different values of V (μl, 5% SDS solution added) and data ($A=1$, $T=33$ mm, $B=18$ mm, and $K=8.79102\times10^{-3}$) into the formula (4) to be plotted. A broken line (panel 1-1 left) passed through the data spots. This fitting implied that the surface tension curve was interpretable by the present hypothesized equilibrium reaction.

For the curve of foam-height (panel 1-1 right in Fig. 8), the hypothesis was as follows: SDS(S) binds with one type of complex WS consisting of S and water W to form another type of complex WSS. the hypothesis was formulated as follows: [WSS]/[WS][S]$=K$, [S]+[WS]+[WSS]$=V$ (amount of SDS added: μl), [WS]+[WSS]$=A$ (amount of water used), [S]=amount of free SDS, [WS]=amount of WS, [WSS]=amount of WSS. These formulae were rearranged as follows:

[WSS]/($A-$[WSS]) $(V-A)=K$ (1) and
[WSS]$=A\ (V-A)/(V-A+1/K)$ (2).

H (foam height ratio)= {M (whole height of foam and aqueous phases in a test tube at a value V)$-B$ (height of aqueous phase at $V=0$)}/{T (whole height of foam and aqueous phases at $V=1000$)$-B$} (3).

[WSS] was further hypothesized to be comparable to value H : [WSS]$=H$. This hypothesis changed formula (2) to $M=(T-B)\ (V-A)A/(V-A+1/K)+B$ (4). Constant value $K=7.84929\times10^{-4}$ was obtained by substituting data ($T=100$ cm, $B=5.5$ cm, and $M=9$ cm at $V=50\ \mu$l of 5% SDS) into the formula (3), and by substituting the calculated value H (=[WSS]), $V=50$, and $A=1$ into formula (1). Different values of M were obtained by substituting different values of V (μl, 5% SDS), and data ($A=1$, $T=100$ cm, $B=5.5$ cm, and $K=7.84929\times10^{-4}$) into the formula (4) to be plotted. The broken line (panel 1-1 right) passed through the plots of experimental data over the range of 0.1 to 100 μl of 5% SDS solution added. Much higher foam-height over the range of 200 μl to 1000 μl was not obtained because of restriction to the length of test tube (18 cm long, 1.5 cm i.d.). The curve of foam height was also found to be roughly interpretable by the present hypothesized equlibrium reaction.

For the delta zone of clear chloroform layers (panel 1-2 in Fig. 8), the hypothesis was as follows: at a high protein concentration range, compared with chloroform, protein P (one portion) binds with chloroform C (one portion) to give a complex PC. Complex PC reacts with SDS (S) and changes to another complex PS releasing chloroform C. This released chloroform forms the delta zone. Namely, the delta zone is an area which is enclosed by two curves of [PC] and [C]. This hypothesis was formulated as follows: [PS][C]/[PC][S]$=K$ (equilibrium constant), [PC]+[PS]$=N$ (amount of protein involved in the sample solution), [PC]+[C]$=M$ (amount of chloroform used), [PS]+[S]$=V$ (amount of SDS added). These formulae were modified as follows:

$(N-M+C)C/(M-C)\ (V-N+M-C)=K$ (1),
[C]$=\mathrm{SQR}\ [\{K\ (2M+V-N)+N-M\}^2/4\ (K-1)^2-KM\ (V-N+M)/(K-1)]+\{K\ (2M+V-N)+N-M\}/2\ (K-1)$ (2), and [PC]$=M-$[C] (3). Constant value $K=0.10011$ was obtained by substituting data: $N=1$ (mg), $V=10$ (μl, 5% SDS), $C=0.09$ (ml), and M $(=2C)=0.18$ (ml) into the formula (1). Different values of [C] (ml) were obtained by substituting different values of V (μl, 5% SDS) and data: $N=1$, M $(=2C)=0.18$, and $K=0.10011$ into the formula (2). Calculated values of [C] gave one broken line (panel 1-2 right) passing through the data spots. Different values of [PC] were obtained by substituting different values of [C] into formula (3). Calculated values of [PC] gave another broken line (panel 1-2 left) passing through the data spots. Both broken lines caused the same delta zone as the experimental one. This fitting suggested that the delta zone was interpretable by the present hypothesized equilibrium reaction. The calculated curve [C] departed upward from the experimental curve over the SDS-range of 10 to 1000. Therefore, the curve [C] seemed to be a curve of complex [SC]. This departure was explained as follows: the present calculation (hypothesis) incorporated both water-soluble and -insoluble chloroform into the complex [SC]. However, the water-soluble chloroform failed to contribute to the formation of complex [SC]. Therefore, the experimental curve was located below the calculated one.

For the trapezoidal zone of clear chloroform layer (panel 2-2 in Fig. 8), the above hypothesis (delta zone) was developed as follows: in a low concentration region of protein compared with that of chloroform, one portion of protein P binds with the r-portion of chloroform to give a complex PCr. This complex reacts with SDS (S) and changes to another complex PS, releasing the r-portion of chloroform. Curves of [PCr] and [C] creates a virtual image of delta zone of clear chloroform layer, based on the hypothesis deschibed dbove (delta zone). The top of the triangular zone is roughly estimated by intersection of two lines extrapolated from both slopes of the trapezoid. The height of the top means the volume of the r-portion of chloroform. Among the r portion of chloroform, one portion (water-insoluble chloroform) serves for the actual formation of the clear chloroform layer. The remaining portion (r-1, water-soluble chloroform) dissolves in water. Consequently, a trapezoidal zone of clear chloroform appears. Calculations were done using the formulae deschibed above. Constant value $K=0.0517799$ was obtained by substituting data: $N=1$ (mg), $V=13$ (μl, 5% SDS), $C=0.36$ (ml), and M $(=2C)=0.72$ (ml) into the formula (1). Different values of [C] (ml) were obtained by substituting different values of V (μl, 5% SDS) and data: $N=1$, M $(=2C)=0.72$ (ml), and $K=0.0517799$ into formula (2). These values gave one broken line (panel 2-2 right) passing through data spots. Different values of [PC] were obtained by substituting different [C] values into formula (3). These values gave another broken line (panel 2-2 left) passing through data spots. The fitting of the calculated trapezoidal zone with the experimental one suggested that the hypothesis was not wrong.

In conclusion, SDS was found to repress gelatinization of chloroform caused by shaking with urine samples. Good separation of clear chloroform from the urine layer was achieved by suitable addition of SDS to urine samples. This addition amount fell in a range where the urine sample began to foam and show a decrease in surface tension. Experimental curves of foam height, surface tension, and clear chloroform layer against different amounts of SDS were superimposed on those derived by hypothetical formulae deduced from the chemical equilibria among SDS, protein, and chloroform.

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